PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF VIBRIO CHOLERAE
ISOLATES FROM A RECENT CHOLERA OUTBREAK IN SENEGAL:
COMPARISON WITH ISOLATES FROM GUINEA-BISSAU


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Abstract. A total of 127 strains of Vibrio cholerae (117 V. cholerae O1 and 10 nonagglutinating strains) isolated from a recent cholera outbreak in Senegal and four strains isolated in Guinea-Bissau (during the survey of a cholera epidemic that occurred 10 months before the Senegalese one) were analyzed. Strains were characterized by conventional methods (biochemical and serologic identification, susceptibility to antimicrobial agents), polymerase chain reaction for genes encoding cholera toxin (CtxA), zonula occludens toxin (Zot), and accessory cholera enterotoxin (Ace), and by ribotyping. Conventional methods showed that all strains of V. cholerae O1 belonged to serotype Ogawa, biotype El Tor and were resistant to the vibriostatic agent O129 (2,4-diamino 6,7-disopropylpteridine phosphate), cotrimoxazole, and chloramphenicol; all strains were sensitive to tetracycline, a drug that has been extensively used in cholera therapy. Most of these V. cholerae O1 (112 strains from Senegal and four strains from Guinea-Bissau) had an intact core region (virulence cassette) and amplified a 564-basepair (bp) fragment of ctxA, a 1083-bp fragment of zot, and a 314-bp fragment of ace. Ribotyping of V. cholerae O1 strains after Bgl I restriction of total DNA revealed that ribotype B5a, which is the predominant ribotype of this seventh pandemic of cholera, was not isolated. Instead, a new ribotype was identified and designated B27 in our data bank. Since O1 isolates from Guinea-Bissau and Senegal have the same biotype, serotype, and ribotype and as the Guinea-Bissau outbreak that preceded the one in Senegal, this emerging ribotype probably came from Guinea-Bissau. Nonagglutinating strains exhibited no resistance to the O129 agent and to the tested antibiotics, they were all negative for virulence cassette, except for one strain with the ctxA and zot genes isolated from a patient with diarrhea, and there was a great variability of ribotypes among these strains. There was no difference between environmental O1 strains isolated from water and strains isolated from patients with cholera, suggesting that fecally contaminated water is an important reservoir for infection.

We are experiencing the seventh pandemic of cholera, which began in 1961 in Southern Celebes, spread throughout the Middle East and Europe, reached Africa via Guinea in 1970, and then extended in 1991 into South America, the one continent previously untouched by cholera in this century. The etiologic agent of this seventh pandemic is Vibrio cholerae O1 biotype El Tor. More recently, in October 1992, a new variant of V. cholerae appeared in a suburban area north of Madras, India. It was designated a new serotype, O139 syn. Bengal, according to the Shimada/Sakazaki typing system.

The most important virulence factor produced by V. cholerae is cholera toxin (CT) an A-B subunit toxin encoded by the ctx gene that catalyzes an ADP ribosylation reaction in epithelial cells. The resulting activation of adenylate cyclase leads to increased intracellular levels of cAMP, alteration of ion transport, and ultimately to secretory diarrhea.

Although CT is responsible for severe dehydrating diarrhea associated with V. cholerae, the search for additional enterotoxins produced by V. cholerae, which has included volunteer studies of genetically engineered Ctx-deleted V. cholerae, has led to the discovery of new toxins. The zonula occludens toxin (Zot) acts on intestinal tight junctions (zonula occludens) to increase intestinal permeability. The accessory cholera enterotoxin (Ace) increases potential difference across intestinal epithelium and alters ion transport. The genes encoding Zot and Ace toxins are located immediately next to the ctx gene on a 4.5-kilbase (kb) dynamic region of the V. cholerae chromosome termed the core region or Virulence cassette. In the past, V. cholerae was characterized by conventional methods using biochemical properties, serologic identification, and antibiotic susceptibility tests. Due to the low discriminatory power of these conventional epidemic tools, newer methods of molecular typing have been developed, including ribotyping polymerase chain reaction (PCR), arbitrary primer PCR, and pulsed-field gel electrophoresis.

Koblavi and others applied the ribotyping system proposed by Grimont and Grimont to V. cholerae using endonuclease Bgl I. The system was later used by Popovic and others with a different nomenclature and types. The ribotyping of 605 strains of V. cholerae revealed 57 RNA gene restriction patterns: 54 ribotypes for V. cholerae O1 and three ribotypes for V. cholerae O139 (Koblavi S, unpublished data). Fields and others described a PCR for the amplification of a 564-basepair (bp) fragment of the cholera toxin subunit A gene (ctxA). In addition, primers have been developed for the amplification of a 1083-bp fragment of the zot gene, as well as a 314-bp fragment of the ace gene.

An epidemic of cholera started in Guinea-Bissau in October 1994 and extended into early 1995. At the end of August 1995, cholera appeared in Dakar, Senegal. As with many developing countries, deficiencies in drinking water supply, food safety, and hygiene practices allowed the disease to spread throughout the country. At the end of the Senegalese epidemic, 12,000 cases of cholera were reported with about 900 fatal cases.

This paper reports the phenotypic and genotypic characteristics of four strains isolated from the cholera epidemic in Guinea-Bissau and 127 strains isolated from the cholera epidemic in Senegal. It includes their biochemical and serologic properties, antibiotic susceptibility, ribotype, and the presence of genes encoding CtxA, Zot, and Ace toxins.
**MATERIALS AND METHODS**

**Bacterial strains.** Four strains from the epidemic in Guin-
ea-Bissau isolated from fecal specimen in 1994 and 127 strains isolated in Senegal (Table 1) in 1995–1996 from fe-
ces, vomit, and the environment (water) were studied (Table 2).

**Phenotypic characterization.** All strains were identified as *V. cholerae* by means of biochemical identification (API 20 E and Biotype 100; BioMerieux, Charbonnieres-Les-
Bains, France). The serologic identification was done using polyclonal, anti-Ogawa, and anti-Inaba antisera. Hemolysin was tested in Columbia agar containing 5% sheep erythro-
cytes. Susceptibility to antimicrobial agents was assayed by two methods: the disk method and the E-test.

For disk method, a culture was grown overnight in trypto casein soy broth and an adequate dilution (0.5 MacFarland standard) was spread on Mueller Hinton agar (Sano-
® Diagnostics Pasteur, Marnes la Coquette, France) as previously described.

The E-test (AB Biodisk Co., Solna, Sweden) is a method based on the diffusion of a continuous concentration of an antimicrobial to determine the minimum inhibitory concentra-
tion (MIC in μg/ml) of individual antimicrobial agents on an agar medium. This test consists of an immeasurable, inert, thin reagent carrier strip, one side of which contains a pre-defined continuous concentration gradient of dried and sta-
bilized drug. The gradient covers a wide range corresponding to 15 log2 dilutions of the conventional MIC procedure. The E-test method for antimicrobial susceptibility is per-
formed as follows. A Mueller-Hinton agar medium is inoc-
ulated with a broth suspension equivalent to 0.5 MacFarland standards prepared by directly inoculating organisms from 24-hr-old agar medium and then using a cotton swab to ap-
ply the suspension. The E-test strips are applied onto the plate and incubated at 37°C for 18 hr. After incubation inhibitory concentrations are seen as a formation of an ellipti-
tical zone of inhibited growth, whose intersection between the value printed on the strip edge and the zone of inhibition is the MIC. A rigorous standardization of operator conditions was established and *Escherichia coli* strain ATCC 25 218 was used as a control.

Standard disks and strips for the following antibiotics were used: ampicillin, amoxicillin plus clavulanic acid, ce-
falotin, cefotoxin, gentamicin, amikacin, tetracycline, chlor-
amphenicol, norfloxacin, flumequin, and trimethoprim plus sulfamethoxazole. The vibriostatic agent 0129 (2,4-diamino 6,7-diisopropylpteridine phosphate), >150 μg per disk, was also tested by the standard disk method.

**Extraction of DNA.** *Vibrio cholerae* strains were inocu-
lated in trypto casein soy broth (Sanofi Diagnostics Pasteur) and incubated in a shaking incubator at 37°C for 18 hr. The cultures were centrifuged (10,000 × g for 10 min) and the pellet was lysed in buffer (0.1 M NaCl, 50 mM disodium EDTA, 0.1 M Tris-HCl, pH 8) with 0.5% (w/v) sodium do-
decyl sulfate 0.5% (w/v), proteinase K (0.5 mg/ml), and RN-
ase (0.8 mg/ml). After precipitation with 6 M NaCl, DNA was extracted with isopropanol, washed with 70% (w/v) eth-
anol, and dissolved in TE buffer (0.1 M Tris, 0.01 M EDTA, pH 8). The DNA was characterized by evaluating its concentration (OD at 260 nm = 1–50 μg/ml of DNA), its purity (1.7 < OD at 260 nm/OD at 280 nm < 2), and its ability to be cleaved by the endonuclease restriction enzyme Eco RI for 1.5 hr at 37°C.

**Ribotyping.** Approximately 2 μg of extracted DNA were cleaved for at least 3 hr at 37°C with *Bgl I* or *Hind III* according to the supplier’s (Boehringer, Mannheim, Ger-
many) instructions. Restriction fragments were separated by electrophoresis in a 0.8% (w/v) agarose gel (Appligene, Illk-
irch, France) in Tris-borate buffer (89 mM Tris, 89 mM bo-
rate, 2 mM EDTA, pH 8.3) for 16 hr at 1.5 V/cm. Restriction fragments of *Citrobacter koseri* strain 32 DNA cleaved with *Mlu I* (Pharmacia-Biotech, Saint-Quentin-Yvelines, France) were used as fragment size markers and were loaded in at least four of the 20 lanes of the gel prior to electrophoresis. Gels were stained with ethidium bromide (Sigma Chemical Co., St. Louis, MO) (2 μg/ml) for 20 min and photographed at 365 nm by UV transilluminaton.

The DNA fragments were depurinated with 0.25 M HCl and transferred to a nylon membrane (Hybond N+; Amer-
sham International, Buckinghamshire, United Kingdom), using an alkali blotting procedure and a vacuum blotter (VacuGene System; Pharmacia-Biotech).

After transfer, the DNA was fixed in an oven at 80°C for 30 min. Prehybridization and hybridization were carried out at 68°C for 1 hr and 58°C for 16 hr, respectively. The probe used for hybridization was a set of five synthetic oligonu-
cleotides corresponding to conserved sequences of 16S and 23S RNA labeled with digoxigenin (Boehringer Mannheim, Mannheim, Germany). Fragments were detected colorimet-

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**TABLE 1**

Origin of Senegalese *Vibrio cholerae* strains

<table>
<thead>
<tr>
<th>Locality</th>
<th><em>V. cholerae</em> O1</th>
<th><em>V. cholerae</em> non O1, non O139</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bakel</td>
<td>11</td>
<td>2</td>
<td>13</td>
</tr>
<tr>
<td>Dakar</td>
<td>58</td>
<td>2</td>
<td>60</td>
</tr>
<tr>
<td>Darou Mousty</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Diofor (Fatrick)</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Diohine (Fatrick)</td>
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<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Diourbel</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Fatick</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Kaolack</td>
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<td>1</td>
</tr>
<tr>
<td>Nioro</td>
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<td>Podor</td>
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<tr>
<td>Porokhane</td>
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<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Richard Toll</td>
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<td>4</td>
</tr>
<tr>
<td>Saint-Louis</td>
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<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Thies</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Touba/Mbacke</td>
<td>11</td>
<td>4</td>
<td>15</td>
</tr>
<tr>
<td>Ziguinchor</td>
<td>6</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>117</strong></td>
<td><strong>10</strong></td>
<td><strong>127</strong></td>
</tr>
</tbody>
</table>

**TABLE 2**

Source of *Vibrio cholerae* strains

<table>
<thead>
<tr>
<th>Source</th>
<th><em>V. cholerae</em> O1</th>
<th><em>V. cholerae</em> non O1, non O139</th>
<th>Total</th>
</tr>
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<tr>
<td>Fecal</td>
<td>108</td>
<td>5</td>
<td>113</td>
</tr>
<tr>
<td>Vomit</td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Environment (water)</td>
<td>5</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>117</strong></td>
<td><strong>10</strong></td>
<td><strong>127</strong></td>
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Characterization of oligonucleotides used for polymerase chain reaction amplification of the ctxA, ace, and zot genes

<table>
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<tr>
<th>Gene*</th>
<th>Oligonucleotide</th>
<th>Sequence</th>
<th>Position</th>
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</thead>
<tbody>
<tr>
<td>ctxA</td>
<td>ctx 1</td>
<td>5′-CGG GCA GAT TCT AGA CCT CCT G-3′</td>
<td>564</td>
</tr>
<tr>
<td></td>
<td>ctx 2</td>
<td>5′-CGA TGA TCT TGG AGC ATT CCC AC-3′</td>
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</tr>
<tr>
<td></td>
<td>ctx probe</td>
<td>5′-CGT TAA TGA TGT AGT ATT GGC ATA-3′</td>
<td></td>
</tr>
<tr>
<td>ace</td>
<td>ace 1</td>
<td>5′-TAA GGA TGT GCT TAT GAT GGA CAC CC-3′</td>
<td>314</td>
</tr>
<tr>
<td></td>
<td>ace 2</td>
<td>5′-CGT GAT GAA TAA AGA TAC TCA TAG-3′</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ace probe</td>
<td>5′-CCG CTT ATC CAA GAG GCT AT-3′</td>
<td></td>
</tr>
<tr>
<td>zot</td>
<td>zot 1</td>
<td>5′-TGG CTT CGT CTG CTT CCG GGG ATT-3′</td>
<td></td>
</tr>
<tr>
<td></td>
<td>zot 2</td>
<td>5′-CAC TTC TAC GCA CAG GCC TGG CG-3′</td>
<td>1083</td>
</tr>
<tr>
<td></td>
<td>zot probe</td>
<td>5′-GCC ACT TTA ACC GGG CCA C-3′</td>
<td></td>
</tr>
</tbody>
</table>

* ctx = cholera toxin subunit A; ace = accessory cholera enterotoxin; zot = zonula occludens toxin.

RESULTS

A total of 127 strains isolated during the recent cholera epidemic in Senegal and four strains from Guinea-Bissau were examined. One hundred seventeen Senegalese strains and four strains from Guinea-Bissau were identified as V. cholerae O1 serotype Ogawa, biotype El Tor by conventional methods. Ten strains were identified as V. cholerae non-agglutinating (no agglutination with the polyvalent anti-serum O1 and antiserum O139). No strain belonging to serotype O139 Bengal was identified.

All V. cholerae O1 strains were hemolytic for sheep erythrocytes, whereas no hemolysis was observed with non-agglutinating strains. Profiles of antibiotic susceptibility of V. cholerae O1 were stable; all strains isolated from the epidemic exhibited resistance to the vibriostatic agent 0129, trimethoprim-sulfamethoxazole, and chloramphenicol. A high level of resistance to chloramphenicol was observed (MIC$_{50}$ = 7.58 µg/L and MIC$_{90}$ = 11.66 mg/L). The MIC$_{50}$ for trimethoprim-sulfamethoxazole was greater than 32 mg/L. All V. cholerae O1 strains showed intermediate resistance to ampicillin, erythromycin, and amoxicillin plus clavulanic acid. They were inhibited by first- and third-generation cephalosporins, particularly ceftriaxone (MIC$_{50}$ = 0.006 mg/L and MIC$_{90}$ = 0.01 mg/L). The other antibiotics tested, including tetracycline, a drug that has been extensively used for the treatment of cholera in Guinea-Bissau, were active. Nonagglutinating strains exhibited no resistance to the tested antibiotics.

Ribotyping of DNA digested with Hind III revealed a characteristic pattern for V. cholerae strains. Nonagglutinating strains were confirmed as V. cholerae by digestion of strain DNA with this enzyme.

The restriction enzyme Bgl I is more discriminating because it produces many profiles with the DNA from V. cholerae strains; therefore, it is more useful for epidemiologic studies. The use of this enzyme has resulted in the description of 53 ribotypes for V. cholerae O1 and three ribotypes for V. cholerae O139 (Koblavi S, unpublished data). From 1937 to 1995, a database consisting of patterns from more than 600 strains of V. cholerae from all continents has been constructed (Koblavi S, unpublished data). The patterns contain four groups of restriction fragments with sizes of approximately 1.5, 4, 10, and 20 kb. The difference among patterns from different samples consisted of several hybridization fragments for each group.

All O1 strains isolated from the Senegalese epidemic and the four strains isolated from Guinea-Bissau in 1994 have the same pattern characterized by the presence of three bands (approximately 10 kb) and one band (approximately 4 kb). This ribotype has never been previously identified. The novel ribotype was designated B27 (Figure 1) and was integrated in our data bank as the 54th RNA gene restriction pattern of V. cholerae O1.

The profiles obtained with nonagglutinating strains do not correspond with those obtained with O1 or O139 strains; this confirms the agglutination results. There was a great variability in ribotypes because three different ribotypes were identified among the 10 nonagglutinating strains (Figure 2) and there was no correlation between the origin of the strains and their ribotype. However, the ribotyping pattern of the nonagglutinating strain with ctxA and zot genes has not been found among the other nonagglutinating strains whereas the two other ribotypes were identified in environmental samples as well as in fecal samples.

Most (96%) of the V. cholerae O1 strains isolated had the complete virulence cassette and amplified a 564-bp fragment of ctxA, a 1,083-bp fragment of zot, and a 314-bp fragment of ace. All of the O1 strains had ctxA, three O1 strains were PCR negative for the ace gene, two were PCR negative for
the zot gene, and one did not have either the zot or ace genes in its genome.

All the nonagglutinating strains were negative for the virulence cassette except for one strain with the ctx and zot genes, which was isolated from a patient with diarrhea.

DISCUSSION

The finding of 0129-resistant strains of V. cholerae O1 emphasizes the need to stop using this vibriostatic agent as a taxonomic tool to distinguish Vibrio spp. from Aeromonas spp.

This study shows that traditional typing systems based on serotype, biotype, and antimicrobial susceptibility are not always able to discriminate between the strains isolated from outbreaks or sporadic cases; all the agglutinating strains in our study were identified as V. cholerae O1, biotype El Tor, serotype Ogawa. As observed by earlier investigators, the rRNA gene restriction pattern is a straightforward approach to the clonal structure of bacterial populations. Also, our study confirms the high degree of reproducibility of ribotyping, as previously reported. Ribotyping with the restriction enzyme Bgl I is a powerful epidemiologic tool for typing V. cholerae O1; application of this method showed that a cholera pandemic is not a single worldwide epidemic due to a single clone but a simultaneous occurrence of several epidemics with several clones involved; V. cholerae O1 biotype El Tor has undergone and continues to undergo evolution since its introduction in west Africa in 1970. The major pattern encountered during this seventh pandemic is ribotype B5a of Koblavi and others corresponding to ribotype 5 of Popovic and others. This ribotype, which represents 55% of African strains (Koblavi S, unpublished data), was isolated during the 1978 Senegalese cholera epidemic; it was associated with ribotype B9 during the 1988 epidemic. This present epidemic is due to the emergence of a new clone never previously identified, which probably came from Guinea-Bissau, and is designated B27 in our data bank. Cholera was found in many west African countries from 1995 to 1996; it would be interesting to look for this emerging ribotype B27 among the strains isolated during these different outbreaks. Most (96%) of the V. cholerae O1 strains have an intact core region (ctx, zot, and ace genes); an intact core region correlates with high toxicity and this explains high morbidity and mortality among malnourished children and patients.

The isolation of nonagglutinating strains during this outbreak does not seem to be of great significance. In two cases, including the nonagglutinating strain with the ctxA and zot genes, nonagglutinating V. cholerae was the only etiologic agent associated with diarrhea, but in three other cases it was isolated in association with another enteropathogenic agent (two associations with Salmonella and one association with enterotoxigenic E. coli).

There was no difference between environmental O1...
strains isolated from water and strains isolated from patients with cholera; this confirms that fecally contaminated water was an important reservoir for infection.


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REFERENCES